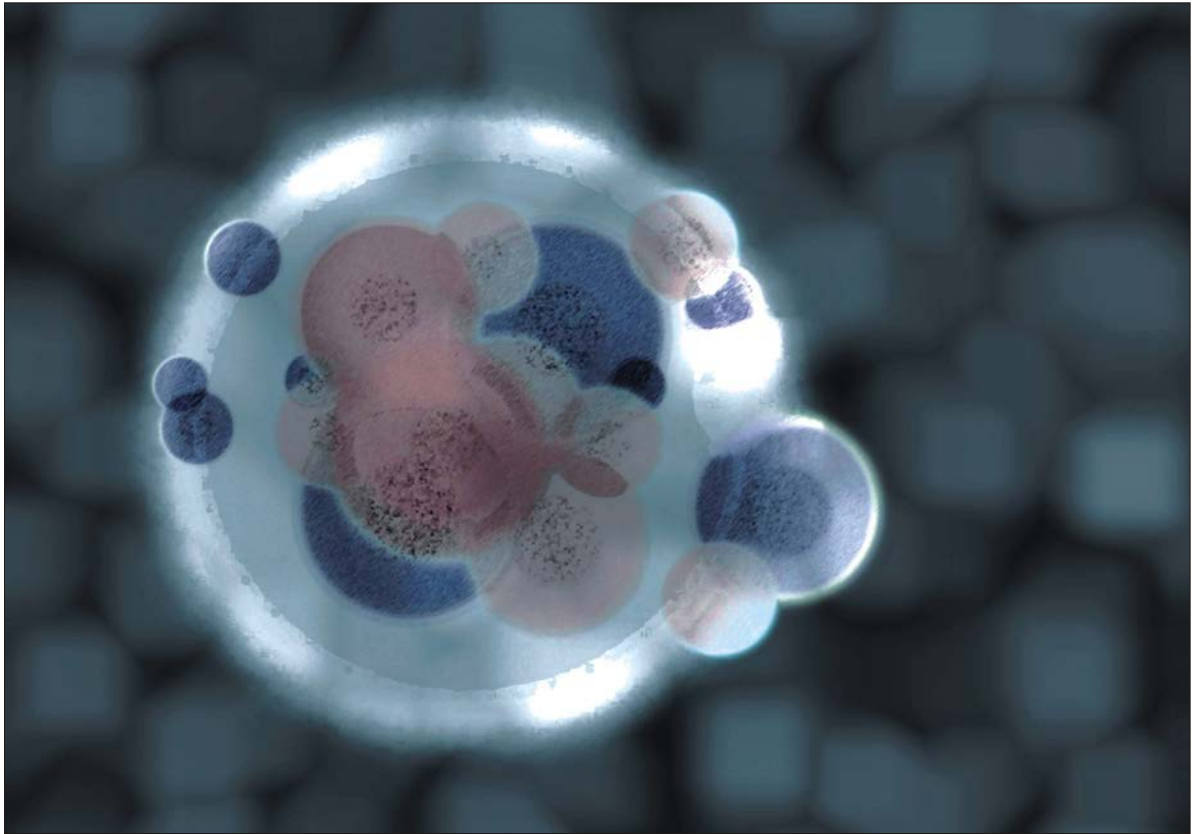

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Additional Possibility of Data Analysis of Enzyme Inhibition and Activation. 5. Comparative Study of Temperature Activation of Calf Alkaline Phosphatase and *Escherichia coli* Alkaline Phosphatase

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Abstract: It was shown that simultaneous account of a course of change in the maximum reaction rate (V) and the Michaelis constant (K_m) by plotting their vector representations in the three-dimensional K_mVt coordinate system allows additional analysis of the dynamics of enzyme temperature activation. It also makes it possible to study the mechanism of enzyme action under varying temperature conditions of technological processes by use of such new parameters of enzyme activation as: a) enzyme activation intensity, b) the overall enzyme activation effect, c) a geometrical portrait of enzyme activation. A comparative study of temperature activation of calf alkaline phosphatase and *Escherichia coli* alkaline phosphatase was performed by conventional and new methods of data processing.

Key words: Alkaline phosphatases, activation energy, intensity activation, geometrical portrait of enzyme activation

INTRODUCTION

In enzymology the solution of many problems is connected with determination of enzyme activation energy (E_a). Consideration of this parameter is required to find out the mechanisms of proceeding of enzymatic reactions and to use the appropriate enzymes at biotechnological pilot plants and under laboratory conditions with the aim to increase the yield of reaction product[1-14].

As a rule, researchers tend to calculate enzyme activation energy (E_a) in the (lg V;1/T) or in (lg k_{cat};1/T) coordinates of Arrhenius by plotting the temperature dependencies of maximum reaction rate (V). A course of change of the second important kinetic parameter of enzyme activation, the Michaelis constant (K_m), is usually not taken into consideration[1-15].

In order to simultaneously take into account the course of change of V and K_m at temperature increase, we propose to use a vector method of data processing in the three-dimensional K_mVt coordinate system (Fig. 4 and 5)[16]. The experiment shows that plotting of such dependencies opens up a new possibility of obtaining additional data on the dynamics of enzyme temperature activation, which is of interest to specialists working in different fields of enzyme application, because this helps to control the proceeding of catalytic enzymatic processes to achieve higher yield of the reaction product.

A comparative study of temperature effect on the course of change in the V and K_m parameters of p-nitrophenylphosphate cleavage by calf alkaline phosphatase and *E. coli* alkaline phosphatase revealed additional possibility of characterizing the mechanisms of reaction proceeding as well as the individual features of the enzyme used.

MATERIALS AND METHODS

Chemicals: Calf alkaline phosphatase (EC 3.1.3.1)-a product of Fluka (Switzerland).

Substrate: para-Nitrophenylposphate (pNPP)-a product of Serva (Germany). Isolation of the periplasmic alkaline

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phosphatase (EC 3.1.3.1.) from *E. coli* is described by Nesmeyanova *et al.*[17].

The concentration of p-nitrophenylphosphate was changed within $1.96{\cdot}10^{-5}$-$0.588{\cdot}10^{-5}$ M for both calf alkaline phosphatase and *E. coli* alkaline phosphatase, the concentration of enzyme was kept constant -1.33 and 0.67 μg mL^{-1}, respectively. Selection of the substrate and enzyme concentrations was stipulated by an interval of minimum error in the determination of K_m and V parameters[18].

The course of pNPP cleavage was recorded by a CF-4DR double-beam spectrophotometer (Optica Milano, Italy).

Reactions were carried out in 0.05 M Tris-HCl buffer, pH 9.0, at ionic strength of 0.1 using NaCl of high purity under constant stirring. Cleavage of the substrate was estimated by absorption increment ($+\Delta A_{400}$) of a solution containing the substrate and the enzyme against a solution of the same composition, but without the enzyme.

Determination of enzyme activity: The initial reaction rate (v) of p-nitrophenylphosphate cleavage was determined by the angle of tangents to initial segments of curves representing the course of reaction proceeding in no less than five parallel experiments. The kinetic parameters (K_m and V) were calculated by plots in the (v^{-1};S^{-1}) coordinates of Lineweaver-Burk. Root-mean-square deviation at five-fold determination was: v = ± 2.5%; K_m, V and E_a = ± 7.5%, l_t and S_t = ± 10%. Enzyme activation energy (E_a) was estimated by plots in the (lg V;1/T) coordinates of Arrhenius (Fig. 3) using the program Sigma Plot Version 4 (USA).

For data processing of enzyme temperature activation in the K_mVt coordinate system, i.e. calculation of the length of L_t vectors and S_t areas overlapped by the mobile end of respective vectors at shift due to temperature effect (Fig. 4 and 5), one needs to use conventional units (c.u.). For that purpose, the following intervals of parameters were employed: t = 10°C = 1 c.u. on the abscissas; $K_m = 1{\cdot}10^{-5}$ M = 1 c.u. on the ordinates and V= 1 μmol pNPP/min μg protein = 1 c.u. on the applicates. This allows estimation of the length of L_t vectors of enzyme activation (l_t, c.u.) by Eq. 4 and the areas overlapped by the mobile end of L_t vectors at temperature increase-by Eq. 1 and 5. As follows from Eq. 4, to calculate the length of the vector for calf alkaline phosphatase at 15°C, the following values of parameters should be used: t = 1.5 c.u., K_m = 1.254 c.u., V = 1.681 c.u. (Table 1), from where it follows that l_{15} = 2.578 c.u. To calculate the S_t area overlapped by the mobile end of L_t vector due to temperature increase t = 15 → 20°C, one should use the first two lines of t, K_m and V parameters: (Table 1, at temperatures: t_1 = 15°C and t_2 = 20°C also expressed in conventional units). It is easy to calculate the length of vectors for enzyme temperature activation (Eq. 4) using a computer equipped with a program for raising to a power and automatic summation of results. As for estimation of S_t areas overlapped by the mobile end of L_t vectors for enzyme temperature activation, it is more advisable to use any of more complex programs, for example, MicroCal Origin, Version 5 (USA) or Eureka (USA) etc. Besides the above procedures, they allow consecutive substitution of K_{m1}, V_1 and t_1 values in the Eq. 1 and 5:

$$S_t = 0.5{\cdot}((K_{m1}{\cdot}V_2-K_{m2}{\cdot}V_1)^2 + (V_1{\cdot}t_2-V_2{\cdot}t_1)^2 + (t_1{\cdot}K_{m2}-t_2{\cdot}K_{m1})^2)^{0.5} \quad (1)$$

As can be easily seen, for calculation of the areas overlapped by L_t vector at shift from one temperature interval (t_1 = 20-25°C) to another one (t_2 = 25-30°C), it is unnecessary to substitute all the parameters of enzyme activation. One may use only temperature values. It is enough to substitute the experimental data pertinent to one temperature value, for example, to replace the data obtained at t_2 = 30°C by those obtained at t_1 = 20°C. The quadratic forms of difference in radicands (Eq. 1) allow data processing in both the increasing ($t_2 > t_1$) and decreasing ($t_2 < t_1$) temperature ranges.

RESULTS AND DISCUSSION

The maximum rate of pNPP cleavage catalyzed by enzymes increased within the temperature range 15-60°C in the case of both alkaline phosphatases (Fig. 1-3 and Table 1).

Conventional data processing of experimental results by plotting temperature dependencies of the course of change in maximum reaction rate allows calculation of enzyme activation energy (E_a) by the slope angle (tg α) of lines:

$$\lg V = -\frac{E_a}{2.303{\cdot}R}\cdot\frac{1}{T} + C \quad (2)$$

in the (lg V;1/T) coordinates of Arrhenius by the equation:

$$E_a = 2.303{\cdot}R{\cdot}tg\alpha = 4.576{\cdot}tg\alpha, \text{ Kcal/mol}, \quad (3)$$

where: tgα -the slope angle of the line (Eq. 2) to the abscissa,
R-the gas constant 1.987 cal/mol,
T-temperature by the Kelvin scale (°K),
C-the integration constant.

Table 1: Temprature dependence of the kinetic parameters of pNPP cleavage; K_m, 10^{-5} M, V, μmol/min.μg.protein, l_t c.u. and S_t, c.u.^{2}; catalysed by calf alkaline phosphatase (Calf) and *E. coli* alkaline phosphatase (*E. coli*)

	Calf			*E. coli*		
Temp (°C)	K_m	V	l_t	K_m	V	l_t
15	1.254	1.681	2.578	0.99	3.174	3.648
20	1.17	2.227	3.214	0.977	4.261	4.807
25	1.08	2.726	3.853	0.954	5.429	6.053
30	1.079	3.332	4.612	0.897	6.124	6.878
35	1.01	3.574	5.103	0.926	7.463	8.295
40	1.157	4.55	6.168	1.02	8.55	9.494
45	1.053	4.995	6.805	1.243	10.95	11.904
50	1.268	5.886	7.826	1.25	12.42	13.447
55	1.443	7.097	9.694	1.545	15.13	16.173
60	1.884	8.36	10.461	1.613	17.16	18.250
Δl, c.u.			7.883			14.603
ΣS_t, c.u.^{2}			8.162			12.507

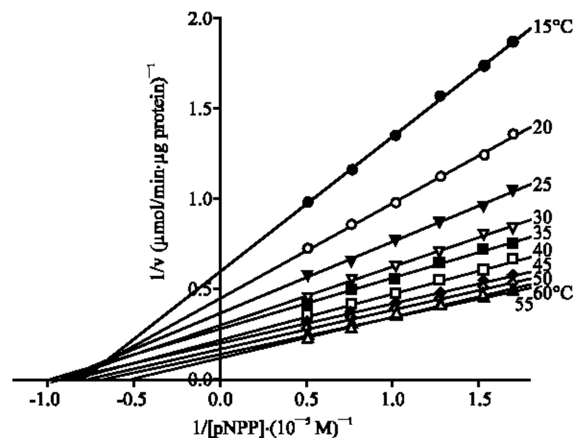

Fig. 1: Plots of activating temperature effect on the initial rate of pNPP cleavage by calf alkaline phosphatase in the double reciprocal coordinates of Lineweaver-Burk. The position of experimental lines at the respective temperature is marked 15°C, 20-60°C

We established that activation energy (E_a) of calf alkaline phosphatase and *E. col* alkaline phosphatase was 6.46 and 6.97 Kcal/mol, respectively, which is in good accord with literature data[1-4]. Insignificant deviation in activation energies (E_a) testifies to similarity in the mechanism of action of both enzymes on p-nitrophenylphosphate, though these enzymes differ in the source of isolation, the molecular mass and functioning at strict temperature regulation (calf alkaline phosphatase) or whithout it (*E. coli.* alkaline phosphatase).

To obtain additional information about the presence or absence of some individual features in the compared enzymes, we considered a simultaneous course of change in the V and K_m parameters as a temperature function in the three-dimensional K_mVt coordinate system. The

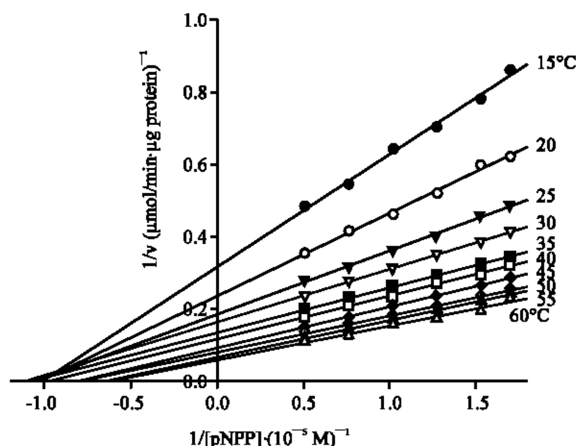

Fig. 2: Plots of activating temperature effect on the initial rate of pNPP cleavage by *E. coli* alkaline phosphatase in the double reciprocal coordinates of Lineweaver-Burk. The position of experimental lines at the respective temperature is marked 15°C, 20-60°C

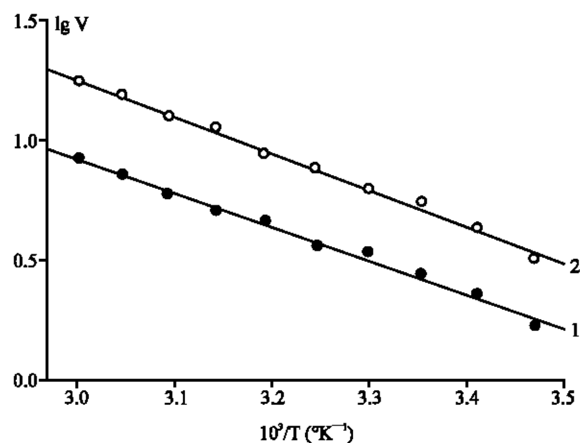

Fig. 3: Reverse temperature (T, °K)$^{-1}$ dependence of the course of change in the logarithm of maximum rate (lg V) of pNPP cleavage by calf alkaline phosphatase (line 1) and *E. coli* alkaline phosphatase (line 2). V (μmol/min μg protein) upon reverse temperature

maximum reaction rate increased at temperature rise, the Michaelis constant also changed (Table 1; Fig. 4, A4, 5 and A5).

Analysis of the position of L_t vectors for enzyme temperature activation in the three-dimensional K_mVt coordinate system reveals (Fig. 4 and 5) that a strictly defined vector representation by length and position in space corresponds to each particular temperature (t) and V and K_m parameter characterizing enzyme activity, i.e., an individual three-dimensional (L_t) vector.

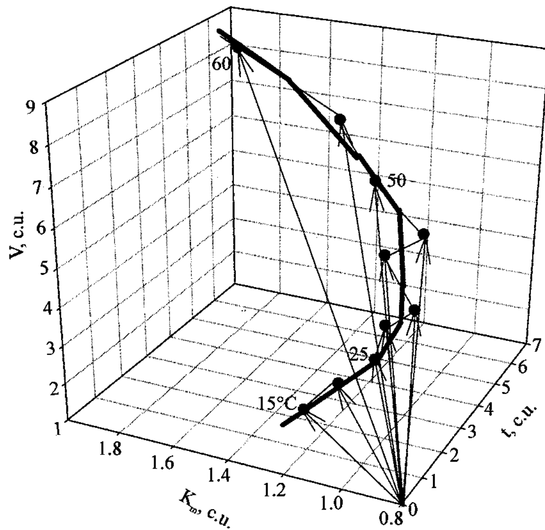

Fig. 4: Temperature dependence of the course of change in the position of L_t vectors for calf alkaline phosphatase activation in the three-dimensional K_mVt coordinate system. The position of L_t vectors for enzyme activation at the respective temperature is marked 15°C, 25, 45 and 60°C. The characteristic (smoothened) line is marked in bold type, the experimental (broken) line obtained by using the K_m, V and t parameters (Table 1) and expressed in c.u., (the broaden variant of Fig. A4)

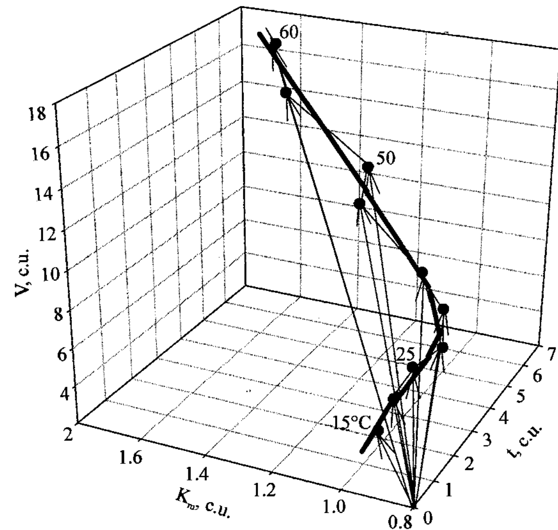

Fig. 5: Temperature dependence of the course of change in the position of L_t vectors for *E. coli* alkaline phosphatase activation in the three-dimensional K_mVt coordinate system. The position of L_t vectors for enzyme activation at the respective temperature is marked 15°C, 25, 45 and 60°C. The characteristic smoothened curve is marked by in bold type, the experimental curve obtained by using the K_m, V and t parameters (Table 1) and expressed in c.u. is a thin curve (the variant of Fig. A5).

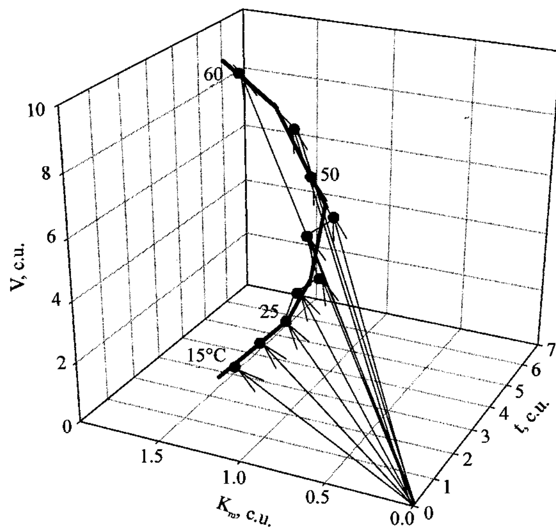

Fig. A4: Initial tree-dimensional K_mVt coordinate system with the data of calf alkaline phosphatase temperature activation

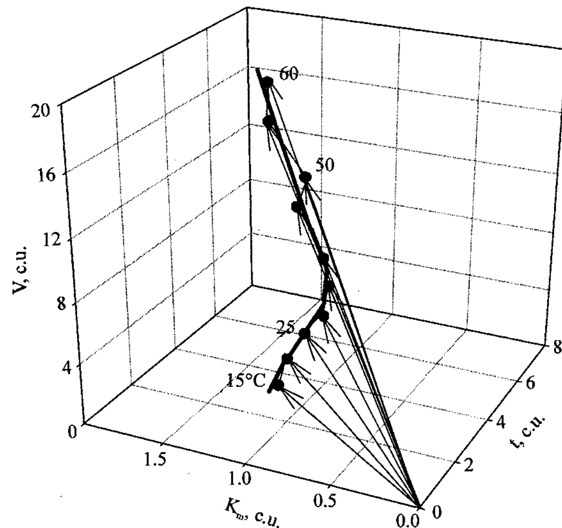

Fig. A5: The tree-dimensional K_mVt coordinate system with the data of *E. coli* alkaline phosphatase temperature activation

Its length at temperature increase:

$$l_t = \sqrt{V^2 + K_m{}^2 + t^2} \quad (4)$$

characterizes the intensity of enzyme temperature activation, the area S_t(c.u.)^2 overlapped by the mobile end of the respective vector at temperature increase:

$$S_t = 0.5 \cdot \left(\begin{vmatrix} K_{m1} & V_1 \\ K_{m2} & V_2 \end{vmatrix}^2 + \begin{vmatrix} V_1 & t_1 \\ V_2 & t_2 \end{vmatrix}^2 + \begin{vmatrix} t_1 & K_{m1} \\ t_2 & K_{m2} \end{vmatrix}^2 \right)^{0,5}, \quad (5)$$

represents the overall effect of enzyme activation in the particular temperature range, the trajectory made by the mobile vector end gives a characteristic curve. As a whole, all these parameters make a geometrical portrait of enzyme temperature activation.

As follows from the experimental data (Table 1; Fig. 4, 5 and 6), temperature has a different activating effect on the tested enzymes

$$\Delta l_t = l_{max}-l_{min}, \quad (6)$$

the developing overall effect of enzyme temperature activation is also different (ΣS_t, c.u.^{2}, Table 1).

Considerable difference is also observed in the forms of geometrical portraits of the enzymes: as seen from the characteristic curve of calf alkaline phosphatase at temperatures above 45°C (Fig.4, the thick curve), the enzyme starts to step-by-step show the features of temperature inactivation and the characteristic bent curve tends to a gradual turn to the left. As for the characteristic bent curve of the second enzyme, *E. coli* alkaline phosphatase (Fig. 5), one can see that the enzyme remains stable at temperature increase even after structural rearrangement within 30-35°C.

Hence, out of the two phosphatases the latter is more suitable to usage in biotechnological processes at high temperatures. It would be more difficult to draw such conclusion only on the basis of conventional experimental data processing (Fig. 1-3 and the Table 1). For example, at t ≈ 55°C the experimental line for calf alkaline phosphatase (60°C) intersects the line (55°C) and is located above it. However, these data are not enough to conclude on higher stability of *E. coli* alkaline phosphatase at temperature activation than of calf alkaline phosphatase under the same conditions (Fig. 2).

The geometrical portraits of enzyme temperature activation (Fig. 4 and 5) that allow to take into account a course of change in all the parameters (V, K_m, l_t, Δl_t, S_t and ΣS_t) as well as the capability of tested enzymes for temperature activation (Δl_t) and the developing overall effect of enzyme activation (Σs_t) (Table 1) permit to report that despite a very close coincidence in the energetic barriers of alkaline phosphatases at cleavage of p-nitrophenylphosphate, they are two distinctive enzymes.

SUPPLEMENT

It is possible to magnify the three-dimensional plots for the course of change in enzyme temperature activation to make them more distinct: the experimental curve (a bent curve) and the characteristic curve (a thick smoothened curve) (Fig. A4 and A5). With this aim, the directing coordinate axes may be extended, for example, by transfer of the start point of vectors (Fig. 4 and 5) to the first

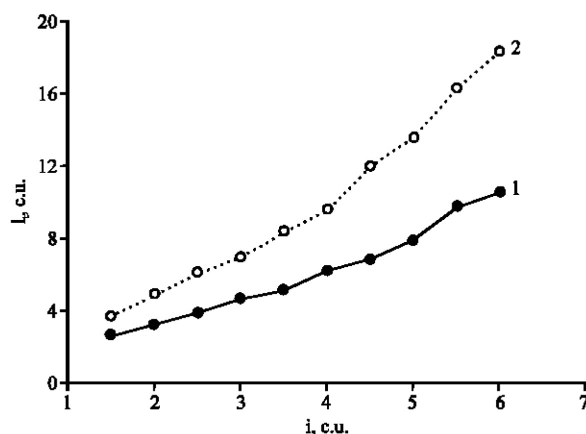

Fig. 6: Temperature dependence of the course of change in the l_t length of vectors for enzyme activation. Curve 1-calf alkaline phosphatase, curve 2- *E. coli* alkaline phosphatase

(or close by) experimental temperature point. In our experiment was taken as (0.;0.8:;1) for calf alkaline phosphatase (Fig. 4) and (0;.0.8;2) for *E. coli* alkaline phosphatase (Fig. 5).

This procedure has no effect either on the course of change in the length (l_t) of vectors or on the areas (S_t) overlapped by the mobile end of vectors (L_t) for enzyme temperature activation. The form of experimental and characteristic curves of enzyme temperature activation remains the same. Actually, only the size is magnified, which makes their outlook more obvious (Fig. 4 and A4; 5 and A5).

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